thin layer chromatography (TLC)-obtained fractions were tested on P3X murine myeloma cell line and a spot showing cytotoxic properties was identified. Such spot was further investigated by mass spectrometry and nuclear magnetic resonance (NMR) techniques. Cell morphology and the array of the cytoskeleton microtubular component were investigated in P3X cells after EC50 biologically active spot treatment.

MATERIALS AND METHODS

Plant material

S. officinalis (*Lamiaceae*) plants were harvested at Tuscia University Botanical Garden in Viterbo (Italy), collected during March–May, and immediately utilized for experimental procedures.

Extraction of plant material and thin layer chromatography

Salvia ethanol extracts and the biologically active spot named "9" were prepared and analyzed by TLC as previously described.[6] The obtained spot contents were tested on murine myeloma cells to evaluate possible cytotoxic properties (see below).

Chemical characterization and mass spectometer investigation

Liquid chromatography–mass spectrometry (LC-MS)/MS analyses were carried out to characterize chemicals using an Ultimate 3000 (Dionex) liquid chromatographer equipped with an UV detector and an autosampler coupled with an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific) equipped with an Ion Max Electrospray Ionization source. The separation of compounds was performed using a C18 150 mm × 2.1 mm i. d. column with 5 µm particles (Phenomenex, Supelco), maintained at 25°C. A volume of 15 µl of each sample was injected into the LC-MS system and the analytes were separated using a linear gradient using 0.1% trifluoroacetic acid (A) and acetonitrile (B). The following linear gradient was used: 0–1 min, 30% B; 1–20 min, 30%–100% B; 20–25 min 100% B; and then back to the initial condition in 2 min. The column was then equilibrated for 6 min. The flow rate was 0.2 mL/min. The UV-VIS detector recorded the spectra at 280 and 360 nm.

The mass spectra were acquired both in positive and in negative mode. The electrospray positive ion mode conditions were source voltage 4.5 kV, heated capillary temperature 275°C, capillary voltage 46.5 V, and tube lens 92 V.

In the negative ion mode, the conditions used were source voltage 3.2 kV, heated capillary temperature 275°C, capillary voltage −45 V, and tube lens −100 V.

The full mass spectra were acquired both in low and high resolution (60,000 FWHM) and were processed using Xcalibur version 2.0 software.

The compounds' identification was performed considering the accurate mass of the protonated or cationized molecules in positive ion mode and of the deprotonated molecules (in negative ion mode) and their fragmentation pathways.

Nuclear magnetic resonance investigations

Mono and bidimensional NMR experiments were performed with a Bruker DRX-600 Avance spectrometer operating at 600.13 MHz for ^{1}H, equipped with an xyz gradient unit. All experiments were performed at 298 K in the methanol (CH_3OD) solution. All the spectra were processed using the Bruker Software XWINNMR.

Murine myeloma cell culture and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

Murine myeloma P3 × 63-Ag8.653 cell line (ATCC, Manasass, VA, USA) is derived from the Balb/c strain of mice. Myeloma cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO_2.

For biological tests, ethanol salvia extracts and the supernatants derived from TLC-scraped spots were concentrated three times in a evaporator; the resulting residues suspended in Milli-Q water (3 volume) and concentrated three times again. Samples were then centrifuged at 14,000 rpm for 3 min, the supernatants filtered through a 0.V22 µm cellulose syringe filter, and stored at −20°C until testing.

For EC50 determination, P3 × 63-Ag8.653 cells were seeded in 96-well plates (10^6 cells/mL) in a total volume of 100 µl/well and after 2 h treated with salvia extract and TLC spots for 24 h. Percentage of viable cells was determined by test (3-[4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide [MTT])[17] and compared with untreated cells. The formazan crystal was dissolved with DMSO for 30 min and absorbance read at 595 nm using the cell microplate spectrophotometer (Sunrise, Tecan).

Scanning and transmission electron microscopy and immunofluorescence analysis

For scanning and transmission electron microscopy analysis and for immunofluorescence analysis, P3X control cells and P3X cells treated for 6 and 20 h with spot B at EC50 concentration were processed.[18]

Statistical analysis

All experiments were carried out in triplicate, and the data analyzed using one-way analysis of variance. $P < 0.05$ was considered statistically significant. EC50 values were carried out by calculating the mean ± standard error of the mean from three different EC50.

RESULTS

To evaluate the effects of treatment of *S. officinalis* leaf extracts on P3X cells, we used the MTT assay, a rapid colorimetric approach used to determine cytotoxicity. By the MTT assay, we found that the extract of *S. officinalis* leaves is highly cytotoxic, with an EC50 of 58.9 ± 9.9 µg/mL after 24 h of treatment. The leaf ethanol extract was subsequently fractionated by TLC and ten spots showing different RF values were isolated. The cytotoxic activity of each single spot was tested and spot 9 showed to be the more effective with an EC50 of 13.1 ± 3.3 µg/mL. The biological active spot 9 was further investigated by LC-MS and it resulted as a

Table 1: Measured masses and elemental composition of Spot 9

Measured mass	Error (ppm)	Elemental composition
345.16980	−2.571	$C_{20}H_{25}O_5$
345.17000	−2.137	$C_{20}H_{25}O_5$
361.20105	−3.011	$C_{21}H_{29}O_5$
389.15961	−2.407	$C_{21}H_{25}O_7$
313.07074	−3.264	$C_{17}H_{13}O_6$
345.16986	−2.571	$C_{20}H_{25}O_5$
359.18546	−2.610	$C_{21}H_{27}O_5$
343.15417	−2.586	$C_{20}H_{23}O_5$
373.20120	−2.030	$C_{22}H_{25}O_5$
315.19556	−3.009	$C_{20}H_{27}O_3$
317.21121	−3.274	$C_{20}H_{29}O_3$
345.20651	−2.066	$C_{21}H_{29}O_4$

mixture of compounds some of them probably derivatives of carnosic acid, a tricyclic diterpene [Table 1].

We further investigated this spot, and separation of the molecules consisting spot 9 was carried out by TLC techniques using a different mobile phase. As a result, five spots were clearly separated and characterized by different RF values. The cytotoxic activity of the separated spots was then analyzed by MTT assay and the spot named B showed an EC50 of 114.8 ± 20.5 μg/mL. The LC-MS analysis of spot B showed the presence of only one component at m/z 345 ([M-H]-) eluting at 18.64 min. The elemental composition measured at resolution 60,000 was $C_{21}H_{29}O_4$. It can be attributed to a methyl derivative of carnosic acid. Unfortunately, it was not possible to ascertain the position of the methyl group; however, the MS/Ms mass spectrum, obtained selecting the ion at m/z 345 as parent ion, evidenced the formation of a product ion at m/z 301 [Figure 1]. The elimination of 44 mass units is attributable to the loss of CO_2, suggesting that the acid group is not esterified. One of the possible candidates could be the 12-methoxy-carnosic acid.

The proton spectrum for a crude extract in methanol solution was recorded at 600 MHz and 298 K [Figure 2a]. The signals at 3.34 and 4.87 ppm were assigned at CH_3OD and H_2O, respectively, whereas the signals at 3.64 and 1.21 ppm correspond to methylene and methyl protons of ethanol. The ^{1}H NMR spectrum of the crude extract showed the aromatic signal at 6.45 ppm, typical for carnosol or carnosic acid derivatives. To confirm such hypothesis, the proton NMR spectrum of carnosic acid in methanol was recorded and compared with the spectrum of the sage leaf extract [Figure 2b]. The experimental comparisons suggested that the extract contained such constituents. The complete assignment ^{1}H peaks [Table 2] were obtained using dqf-COSY spectrum (data not show) recorded at 600 MHz and 298 K, following the approach of Cuvelier *et al*., 1994,[19] for an active antioxidant compound in *S. officinalis*.

In the leaf extract, the H6ax showed a downfield shift with respect to standard compound, occurring at 2.86 ppm, whereas the H6eq remained at 2.79 ppm, thus confirming the presence of carnosic acid derivatives such as methyl carnosate in the extract. This was further examined by proton NMR spectrum in the region between 0.6 and 1.4 ppm [Figure 3]. The major difference between carnosic acid and methyl carnosate is the presence of a methyl group, which produces an additional signal in the up-field region. However, the spectrum showed a triplet at 1.21 ppm that was attributed to the methyl group of the ethanol solvent and that do not allow to identify the methyl signal from the methyl carnosate [Figure 4].

To understand on the biological effects induced by the spot B, P3X cells were treated for different time points (6 and 20 h) with EC50 concentration[20] and then prepared for scanning electron microscopy, transmission electron microscopy, and immunofluorescence

Table 2: ^{1}H chemical shift of carnosic acid in the *Salvia officinalis* leaf extract

H-atom	Chemical shift (ppm)
14	6.45
1eq	3.45
15	3.22
6ax	2.86
6eq	2.79
7eq	2.78
2ax	1.87
5	1.77
2eq	1.55
3ax	1.48
7ax	1.38
3eq	1.32
16	1.18
17	1.17
18	1.02
19	1.00
1ax	0.98

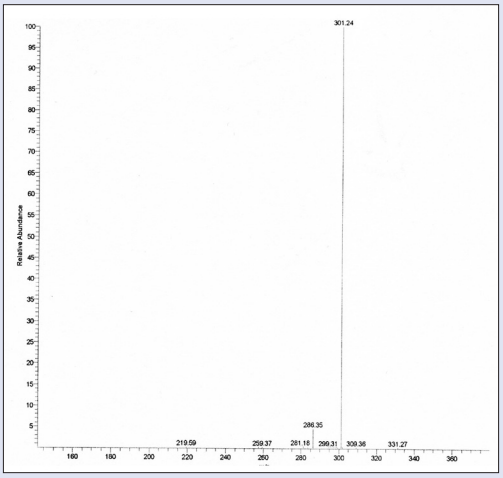

Figure 1: The MS/Ms mass spectrum evidenced the formation of a product ion at m/z 301

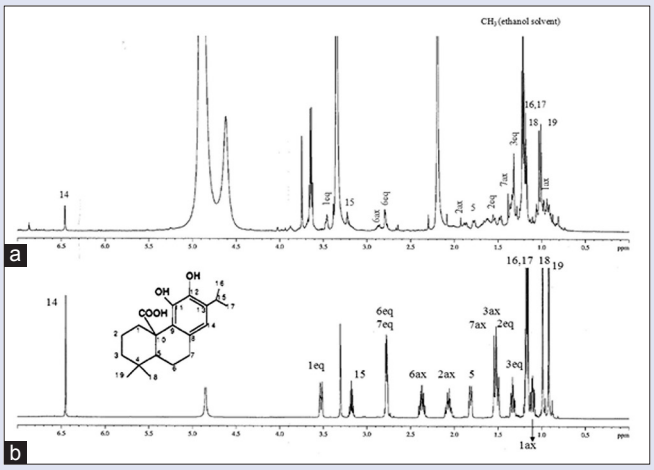

Figure 2: Nuclear magnetic resonance proton spectra of (a) *S. officinalis* leaf extract in CH_3OD solution; (b) carnosic acid in CH_3OD solution at 600 MHz and 298 K

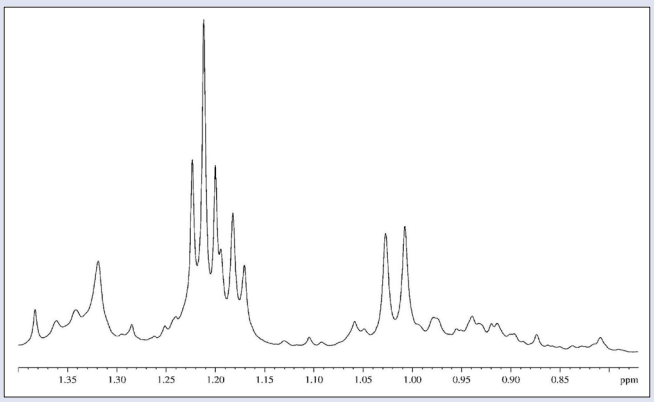

Figure 3: Nuclear magnetic resonance proton spectrum in the region 1.40–0.70 ppm of *Salvia officinalis* leaf extract in CH_3OD solution

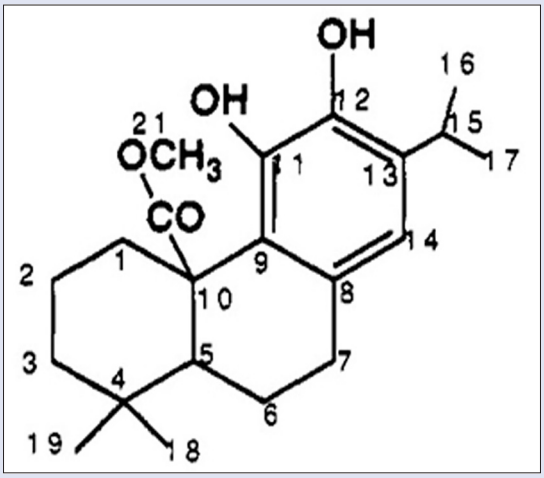

Figure 4: Chemical structure of methyl carnosate

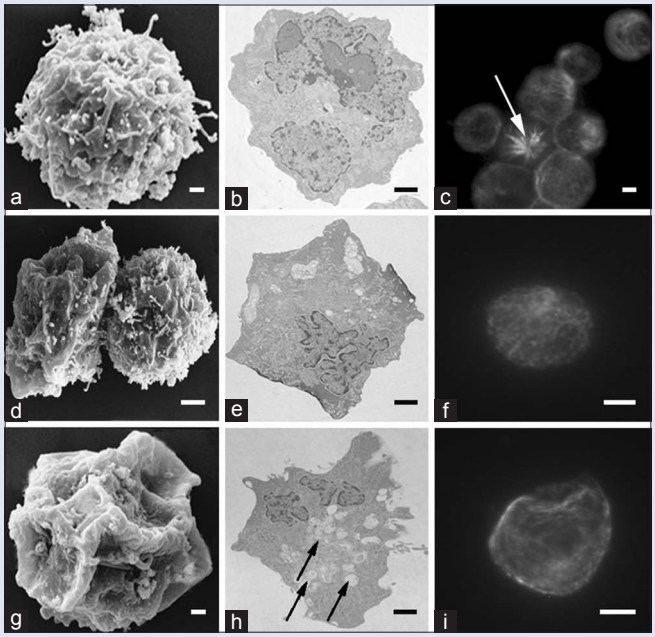

Figure 5: Murine myeloma control cells investigated by scanning electron microscopy (a), by transmission electron microscopy (b) and by immunofluorescence technique (c); 6 h treated murine myeloma cells and investigated by scanning electron microscopy (d), by transmission electron microscopy (e) and by immunofluorescence technique (f); 20 h treated murine myeloma cells and investigated by scanning electron microscopy (g), by transmission electron microscopy (h) and by immunofluorescence technique (i) Bars: a, g = 1 μm; b, e, h = 2 μm; c, d, f, i = 5 μm

investigations. Control murine myeloma cells showed an irregular spherical shape and many short filamentous structures on the cell surface [Figure 5a]. The nucleus was large, and in the cytoplasm, a set of organelles and many vesicles were detectable [Figure 5b]. Microtubules showed a regular distribution, and the microtubule organizing center was clearly visible [Figure 5c, arrow]. After 6 h of spot B treatment, most of the cells seemed to be unaffected and appeared similar to control cells [Figures 5d-e]; only a minority of them showed large invaginations on the cell surface, and the microtubular array resulted unaffected although some bright spots, probably formed by unpolymerized tubulin, were present in the cytoplasm [Figure 5f]. After 20 h, many cells resulted consistently affected by EC50 spot B treatment and survived cells showed an irregular shape with the loss of cell protrusions [Figure 5g], and in the cytoplasm, a membrane system with disordered distribution and many vacuoles occurred [Figure 5h, arrows]. Some microtubules were still present within cells although the presence of fluorescent spots pointed out a consistent microtubule depolymerization process [Figure 5i].

DISCUSSION

Various anticancer agents were originally developed from natural sources,[21,22] and epidemiological investigations and laboratory studies have indicated that bioactive natural compounds play an important role in the treatment of many cancers.[23,24] In this report, sage leaf extract was shown to inhibit the proliferation of P3X murine myeloma cells. EC50 resulted by MTT assays revealed that the spot named 9, isolated by TLC separations, has a strong antiproliferative effect. Further separation of molecules, consisting the spot 9, allowed us to identify spot B as the main responsible of antiproliferative activity on murine myeloma cells.

LC-MS and NMR techniques allowed us to identify methyl carnosate, a methyl derivative of carnosic acid, as the main component of spot B and its major candidate in antiproliferative activity. Methyl carnosate belongs to abietane diterpenoid family, a class of diterpene compounds which show important biological activities such as antimicrobial, antioxidant, antiviral, anti-inflammatory, and antitumor activities.[25] Among dipertens, carnosic acid and its derivate carnosol have well-documented biological properties such as anti-inflammatory[26] and anticancer properties.[27-35] In particular, carnosol was shown to induce apoptotic cell death in different human leukemia cell lines, affecting the mitochondria membranes and reducing Bcl-2 expression without cause significant cytotoxicity on normal PBMCs.[36]

In our hands, spot B containing methyl carnosate was able to induce changes in cellular shape and cytoplasmic organization of murine myeloma cells survived to the treatment. The EC50 value of the spot 9 is approximately ten times lower the EC50 value of the spot B, in which methyl carnosate is the more abundant molecule. Probably, spot 9 cytotoxicity is due to the presence of the other molecules which act synergically inducing a stronger phenotype. Immunofluorescence assays carried out at different time points after spot B treatment highlight a low structural damage to the microtubular apparatus, thus confirming the hypothesis that spot B does not interfere directly with microtubules, and that the microtubule structural changes are related and/or dependent on affections occurring to other cytoplasmic targets.

CONCLUSIONS

Our findings contribute to enlarge the knowledge on the biological properties of *S. officinalis* and identify its component methyl carnosate as an attractive candidate for cancer prevention in addition to antibacterial properties previously identified.[6]

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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